

The Bovine Cornea Opacity and Permeability Test

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Background

An in vitro test for the development of corneal opacity in response to ocular irritants was first proposed by Muir (1984). This test required the clamping of a bovine cornea between two chambers filled with physiologic saline and measuring the transmittance of white light through the cornea using the voltage output of a photocell. This was followed by exposure of the epithelial surface of the cornea to a surfactant and subsequent measurement of light transmittance, with a decrease in transmittance due to development of corneal opacity interpreted as a toxic effect of the surfactant on the cornea.

Subsequent to the work of Muir this testing method was developed and refined by Gautheron et al. (1992). They have described in detail the chambers for mounting the corneas, the opacimeter for measuring light transmission and the experimental protocols for exposure of corneas to test materials. This group also added the measurement of corneal permeability to the protocol. This involves introduction of a sodium fluorescein solution into the epithelial side of the chamber and measurement by absorbance at 490 nm of the amount fluorescein penetrating the cornea into the endothelial compartment. This initial study showed that the toxicity of a large number of compounds could be measured based on development of corneal opacity and increased permeability and that a reasonable correlation existed with Draize data for these compounds. Subsequent multilaboratory validation studies have led to significant acceptance of the "BCOP" assay by industry (Gautheron et al. 1994; Sina 1994; Christian and Diener 1996). Recently, Casterton et al (1996) have proposed a modification of the detection system for the BCOP assay in which the corneal chambers are mounted in a spectrophotometer and the development of opacity is detected by measuring absorbance at 570 nm.

Critique and Suggestions for Improvements

Among the many assays that have been proposed for in vitro ocular toxicity testing, the BCOP assay has several strengths and advantages. It has high relevance to the intact eye given that it utilizes a fresh, intact cornea, the tissue that is weighted most heavily in in vivo tests. The response of the cornea to known irritants using this technique appears to be similar in many cases to the in vivo response and, the limitations of the Draize notwithstanding, it appears to be correlated with historical in vivo data. Finally, in the interests of reducing the use of laboratory animals in toxicology and developing tests which may be considered to be more humane than in vivo tests, the BCOP uses tissue that is inexpensive and readily available from slaughter houses.

The BCOP has a number weaknesses and technical improvements can be made so that the assay is done under more physiologic conditions than those provided by the current protocol. While the current method may be largely adequate for screening of compounds for possible corneal damage or irritancy, it is important that the assay be optimized if it is to gain regulatory acceptance. Isolated, perfused corneas have long been used in ophthalmic research to study many aspect of corneal structure and function, including ion transport processes, electrophysiology, maintenance of transparency, drug penetration and toxicity, and effects of intraocular irrigating solutions. This research has resulted in a voluminous literature that emphasizes the meticulous care that must be taken in preparing, mounting and providing appropriate physiological conditions for the in vitro cornea in order to obtain reliable and relevant data. Curiously, the reference lists of the articles on the BCOP assay that appear in the toxicology literature show little evidence that the authors have drawn upon this database. Following are a number of suggestions for improvements and modifications to the BCOP assay that may potentially improve the quality of the data obtained, provide additional information concerning effects of test substances on the cornea, and allow the test to stand alone without reference to historical Draize data. This critique is based on a reading of the literature, direct observation of the BCOP assay by the author in the laboratory of Philip Casterton at Amway Corporation, and discussions with Dr. Henry Edelhauser at Emory University.

The Corneal Holder

The corneal holder as illustrated by Gautheron et al (1992) and Casterton et al (1996) clamps the cornea between two Lucite half cells. Although a ring of sclera remains around the cornea, the holder clamps directly onto the corneal tissue because the opening on the holder is only 1.7 cm in diameter compared to the 3 cm diameter of the bovine cornea. This causes a significant amount of "edge damage" to the cornea as evidenced by a 2-3 mm ring of edema and opacification that develops around the edge of the cornea during 1-2 hr incubation under control conditions. This is evidence of damage to the endothelial and epithelial cell layers. In the Gautheron opacitometer this may have an effect on opacity readings. In the Casterton method absorbance is measured through a small area in the center of the cornea so edge damage may not have a direct effect on absorbance readings; however, edge damage to the epithelial layer could provide access for a test substance to the stroma and endothelium of the cornea that might not otherwise be able to penetrate the epithelial barrier, resulting in a false positive. Edge damage does not apparently affect BCOP assay permeability measurements as the amount of fluorescein that penetrates the cornea and reaches the 5 ml volume of the endothelial chamber is below the limits of detection when fluorescein is measure by absorbance at 490 nm. The edge damage might, however, become a significant factor if a fluorimeter is used for detection or if substances are tested that require greater sensitivity.

It is therefore suggested that the corneal holder be redesigned so that the sclera is clamped with no contact of the holder with the corneal surfaces. Such a holder for bovine corneas has been diagramed by Andermann (1995) and by Johnson and Tschumper (1987) for human corneas.

Clamping on a circular ring of sclera will also eliminate that wrinkling that occurs when the elliptical bovine cornea is clamped into the circular opening of the holder.

Physiologic Saline

The medium currently used in the BCOP assay is the cell culture medium, Minimum Essential Medium (MEM), with 1% fetal bovine serum. While MEM will probably not have a detrimental effect on the cornea, it is certainly not necessary to use a cell culture medium for this assay and the FBS may definitely be eliminated. This will reduce the cost of the assay and eliminate the foaming that occurs during medium changes due to the presence of protein. A great deal of ophthalmic research has been directed towards determining the optimum medium for maintenance of a normally functioning cornea. The ultimate goal of this work was to develop the best solution for irrigating the anterior chamber of the eye and the corneal endothelium during extended periods of intraocular surgery. This resulted in the development of a balanced salt solution with glutathione, glucose and bicarbonate as described McCarey et al (1973) and Edelhauser et al. (1975, 1978). This solution, which would provide optimal conditions for the BCOP assay, may be prepared in the laboratory or purchased commercially as BSS PLUS[®] (Alcon Laboratories, Inc., Fort Worth ,TX).

A second concern about the BCOP protocol is that the solution is static in the chambers of the corneal holder during the assay. This will lead to lack of oxygen, a drop in pH and build up of metabolites. It is standard practice in corneal research to perfuse the chambers of the corneal apparatus with fresh oxygenated medium, equilibrated with 5% CO₂. The existing corneal holders for the BCOP assay could easily be modified to allow such perfusion.

Finally, the permeability protocol of BCOP assay requires that a 0.4% sodium fluorescein be applied to the epithelial surface in Dulbecco's phosphate buffered saline (PBS). The rationale for the change from MEM to a solution containing only sodium, chloride and phosphate ions is unclear. The glaring omission here is the lack of calcium ions in PBS which are required for maintenance of tight junctions between epithelial cells. Bathing the epithelium in PBS could therefore increase the permeability the epithelium to fluorescein leading to high permeability readings. The same solution must be used throughout the study.

Temperature

The BCOP protocol calls for incubation of the corneas at 32°C for a 1 hr equilibration period. This is accomplished by immersing the corneal holders in a water bath. This temperature is not maintained during the test material exposure period, which according to various reports lasts between 10 and 60 minutes and most often is conducted at room temperature (Gautheron et al 1992, 1994; Sina 1994; Casterton et al, 1996). The cornea is then returned to the water bath for 2 hr before opacity is measured. The 1.5 hr exposure to fluorescein, on the other hand, is conducted at 32°C. The temperature of the cornea therefore fluctuates during the experiment.

Dikstein and Maurice established in 1972 that maintenance of corneal transparency is an active, metabolic process that is temperature sensitive. During the BCOP assay it is essential that the temperature be maintained at physiologic levels (32-35°C) throughout the study so that changes in corneal hydration and transparency do not occur that are unrelated to effects of a test material. Water jacketed corneal holders or a means of suspending the holders in a water bath while maintaining access to the epithelial chambers should be designed. The Casterton chambers with removable end windows would lend themselves well to the latter modification.

What Does Corneal Opacity Mean?

The BCOP assay classifies materials as non-, mild, moderate or severe irritants based on the development and degree of corneal opacity but does not differentiate compounds on the basis of the mechanism by which the loss of transparency is induced. A corrosive material, such as a strong base, will denature and coagulate protein leading to irreversible corneal opacity. A loss of transparency may also be due to corneal edema. This increase in hydration may be due to loss of the epithelial or endothelial barrier function or inhibition of cellular transport processes. The BCOP assay does not address this issue. Casterton et al state, "It is reasoned that when attempting to classify eye irritation potential into three broad categories (mild, moderate, severe), it matters more that the measured damage actually happened rather than why it happened". If the BCOP assay is to be a useful replacement for animal testing and stand alone without regards to historical data, the "why" question must be answered. This is required to give the test predictive value since recovery may be possible if loss of transparency is due to edema. The ability to recover from corneal edema is, however, dependent on the cause of the edema. The BCOP currently does not answer such questions but certain modifications and additions to the protocol can be made to address these issues.

This issue has relevance to the evaluation of Casterton's proposed use of measurement of absorbance at 570 nm. This method has the advantage of being well defined, precise and reproducible. It is not clear from the publication (Casterton et al. 1996) whether the absorbance at 570 nm is unique or whether light scatter and opacity can vary depending on the wave length used. It is also conceivable that the cornea could absorb light at 570 nm differently depending on whether the opacity is caused by edema or chemical damage to proteins. This matter deserves further investigation for validation of the spectrophotometric BCOP assay.

It is also possible that a clinically significant degree of edema might not be detected by an opacimeter or by absorbance. To answer this question and to determine whether opacity is caused by edema or corrosive damage (or some combination of processes) is suggested that direct measurements of corneal thickness and hydration be added to the BCOP assay. Using the Casterton chambers with removable end windows corneal thickness could easily be monitored throughout the experimental period using an ultrasonic pachymeter. At the end of the experiment corneal hydration should be determined by measuring wet and dry weights of 8 mm corneal punches. This value would be compared to the hydration of paired control corneas and untreated fresh corneas. Normal corneal hydration should be about 78%.

Finally various histologic methods can be used to evaluate corneas at the end of the BCOP assay. An especially useful method for analysis of unfixed tissue is alizarin red staining of the corneal endothelium (Means et al. 1995) This will allow immediate determination of whether an increase in corneal hydration is due to loss of endothelial cells.

Conclusion

The above discussion of the BCOP assay may be considered to be a physiologist's view of an ideal toxicologic method. The suggested measures may seem extreme to the toxicologist who is primarily interested in screening compounds rather than gaining an in depth understanding mechanisms of irritative responses. For the BCOP assay to gain acceptance by the broader scientific community it is essential that attention be paid to detail as outlined in this review.

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